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Tumor necrosis factor α stimulates sphingomyelinase through the 55 kDa receptor in HL-60 cells

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Tumor necrosis factor α (TNFα) stimulated rapid (seconds) hydrolysis of sphingomyelin in HL-60 cells, formation of phosphocholine (PCho) and a decrease in choline. The response to TNFα was concentration dependent with a maximal effect at 3–10 nM. The monoclonal antibody (mAb), htr-9, which behaves as an agonist at the 55 kDa subtype of the TNF receptor, also stimulated sphingomyelin hydrolysis in intact cells. In contrast, the mAb, utr-1, which behaves as an antagonist at the 75 kDa receptor subtype, had no effect on sphingomyelin hydrolysis either on its own or in the presence of TNFα. In addition, htr-9 or TNFα stimulated hydrolysis of sphingomyelin in a membrane fraction of HL-60 cells. These results are consistent with a role of sphingomyelin hydrolysis as an early event in the signalling mechanism of TNFα, and suggest that this pathway is activated through the 55 kDa subtype of the TNF receptor.

Tumor necrosis factor α; Sphingomyelinase

1. INTRODUCTION

Several metabolites of sphingolipids are biologically active molecules that affect a variety of cellular responses [1] including inhibition of growth factor action [2,3], modulation of receptor function [4], and antagonism of phorbol ester-induced responses [5,6]. Many of these actions appear to be mediated through inhibition of protein kinase C by sphingosine [1], although a ceramide-activated protein kinase has also been identified [7]. Recent evidence suggests that sphingomyelin and its metabolites may play an important role in signal transduction.

Tumor necrosis factor α (TNFα) is a monocyte/macrophage-derived cytokine with a wide variety of biological activities. TNFα has a cytotoxic/cytostatic effect on many transformed cells [8,9], acts as growth factor for fibroblasts and chondrocytes [8,10], stimulates secretion of prostaglandin E_2 in synovial cells [11] and activates a cyclooxygenase in osteoblasts [12]. The specific binding of TNFα to cell surface receptors appears to be the first step for mediating these events. A variety of cells express specific high-affinity binding sites for TNFα, and two major types of receptor have been identified with apparent molecular mass of 75 kDa and 55 kDa [13,14].

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Abbreviations: TNFα, tumor necrosis factor α; TLC, thin layer chromatography; mAb, monoclonal antibody; GroPCho, glycerophosphocholine; PCho, phosphocholine.

Recently, TNFα has been shown to activate a neutral sphingomyelinase to generate ceramide [15,16] and it has been suggested that this has an important role in the regulation of many of the above responses. However, it is not known which of the two receptor subtypes mediates activation of sphingomyelinase. The present study has sought to address this question using HL-60 cells which express similar amounts of the 55 and 75 kDa receptors. Monoclonal antibodies (mAbs), utr-1 and htr-9, which were raised against the 75 kDa and 55 kDa receptors, respectively [17], were used to determine which receptor mediates the effect of TNFα on sphingomyelin breakdown.

2. MATERIALS AND METHODS

2.1. *Materials*

TNFα was from British Bio-technology Limited or was a kind gift from Dr. R. Foulkes, Celltech. [*methyl*-^{3}H]Choline (80 Ci/mmol) was from Amersham Corp. Silica Gel 60G plates were purchased from Merck. Monoclonal anti-TNFα receptor antibodies, utr-1 and htr-9, were generously provided by Dr. M. Brockhaus. HL-60 cells were generously provided by Dr. C. Madin (Department of Pathology, Oxford University). RPMI 1640 medium was from Gibco. Other reagents were of the highest grade available.

2.2. *Cell culture and labelling*

HL-60 cells were grown in RPMI 1640 medium containing 10% fetal calf serum at 37°C under 5% CO_2 in air. For the labelling, cells were maintained with [^{3}H]choline (0.5 μCi/ml) in culture medium for 48 h. Following labelling, the cells were twice washed with Hank's buffered saline and then incubated with or without TNFα at 20°C or 37°C for the indicated time. For the experiments using mAb utr-1, cells were incubated with utr-1 (10 μg/ml) for 1 h and then incubated with TNFα. At the end of the incubation, 560 μl of chloroform/methanol/water (100:200:2, v/v) was added and phases separated by adding 190 μl of chloroform and water to give a final ratio of 2:2:1 (chloroform/meth-

anol/water). The upper aqueous phase was taken for analysis of the water-soluble choline-containing metabolites. An aliquot of the lower phase was dried down for analysis of lipids.

2.3. *Ion-exchange chromatography*

Dowex-50-WH$^+$ (500 μl) was washed with 1 M HCl followed by distilled water until a constant pH of approx. 5.5 was obtained. The upper aqueous phase was loaded onto each column. The run-through and a 15 ml water wash were collected together; this fraction contains glycerophosphocholine (GroPCho) and phosphocholine (PCho). The choline fraction was eluted by addition of 6 ml 1 M HCl [18]. An aliquot (6 ml) of each fraction was counted for radioactivity following addition of scintillant.

2.4. *Analysis of lipid breakdown*

The lipids were separated using chloroform/methanol/acetone/acetic acid/water (6:2:8:2:1, v/v) on silica gel 60 TLC plates. After visualizing with iodine vapor, each spot corresponding to phosphatidylcholine and sphingomyelin, assessed by co-elution with authentic phospholipids, was scraped and counted for radioactivity following addition of scintillant.

2.5. *Membrane preparation of HL-60 cells*

Cells, prelabelled for 48 h with [^{3}H]choline, were suspended in a solution of 5 mM $MgCl_2$, 100 mM KCl, 20 mM HEPES (pH 7.4) and 2 mM glucose, and homogenized by sonication at 4°C. The resulting homogenate was centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was resuspended in the same buffer and incubated with or without TNFα (10 nM) or mAb htr-9 (10 μg/ml) for the indicated time at 37°C.

2.6. *Statistical analysis*

Experiments were performed in quadruplicate and on at least two occasions. Results are shown as means ± S.E.M. and statistical significance was indicated by Student's t-test with $P < 0.05$ taken as the level of significance.

3. RESULTS

3.1. *Effect of TNFα on [^{3}H]choline-labelled metabolites*

The enzyme sphingomyelinase acts on sphingomyelin to produce ceramide and PCho. Recently, several reports have suggested that TNFα stimulates sphingomyelin hydrolysis by activation of neutral sphingomyelinase [15,16]. In the present study, we have examined the effect of TNFα on [^{3}H]PCho release in HL-60 cells prelabelled with [^{3}H]choline. TNFα stimulated a rapid increased release in the fraction containing [^{3}H]GroPCho and [^{3}H]PCho in a concentration-dependent manner; it was not possible to separate [^{3}H]GroPCho and [^{3}H]PCho on Dowex-50-WH$^+$ columns. The time–course of the stimulation was very rapid at 37°C (not shown), and so all experiments using intact cells were performed at 20°C where maximal increases were obtained after 5–10 min (Fig. 1A). The maximally effective concentration of TNFα for release of [^{3}H]GroPCho/[^{3}H]PCho was 3–10 nM (Fig. 1C). In contrast, release of [^{3}H]choline was decreased by TNFα in a time- and dose-dependent manner in intact HL-60 cells (Fig. 1B and D). The time–courses and concentration curves for [^{3}H]choline release were similar to those for stimulation of [^{3}H]GroPCho/[^{3}H]PCho formation.

In parallel with the above observations, TNFα stimulated a concentration- and time-dependent decrease in [^{3}H]sphingomyelin and [^{3}H]phosphatidylcholine in intact cells. A maximal effect was at 10 nM TNFα and is shown in Table I along with changes in choline and GroPCho/PCho.

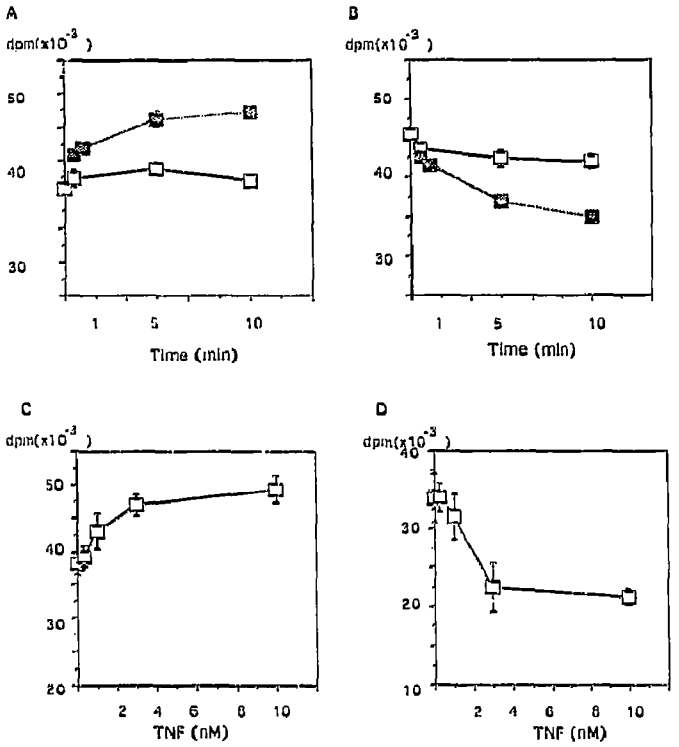

Fig. 1. Effect of TNFα on [^{3}H]choline-labelled metabolites. (A and B) Time-course. The cells were labelled with [^{3}H]choline (0.5 μCi/ml) for 48 h and then stimulated with (■) or without (□) 10 nM TNFα at 20°C. The results are the mean ± S.E.M. of two experiments, performed in quadruplicate. (C and D) Dose dependence. The cells were labelled with [^{3}H]choline as above and stimulated with TNFα for 10 min. The results are the mean ± S.E.M. from one typical experiment, performed in quadruplicate, which is representative of two other similar studies. A and C, GroPCho/PCho fraction; B and D, choline fraction.

Table I

Effect of mAb utr-1 and htr-9 on [^{3}H]choline-labelled metabolites and [^{3}H]choline-labelled lipids

	GroPCho+PCho	Cho	Sphingomyelin	Phosphatidylcholine
TNF	118.5 ± 0.6*	79.3 ± 3.0*	84.0 ± 1.0*	90.3 ± 2.1*
utr-1	103.6 ± 1.2	94.6 ± 1.8	95.6 ± 1.2	99.4 ± 1.8
utr-1+TNF	120.2 ± 0.6*	71.8 ± 3.7*	81.5 ± 0.2*	91.0 ± 2.3*
htr-9	119.2 ± 1.5*	77.8 ± 2.2*	77.1 ± 2.2*	89.7 ± 4.5*

The cells were prelabelled with [^{3}H]choline for 48 h and then incubated with or without mAb utr-1 (10 μg/ml) for 1 h followed by stimulation with TNFα (10 nM) or htr-9 (10 μg/ml) for 10 min at 37°C. Results are shown as % control values (=100% and are the mean ± S.E.M. of two experiments, performed in quadruplicate. *$P < 0.05$ relative to control value.

3.2. *Effect of mAb utr-1 and htr-9 on [^{3}H]choline-labelled metabolites*

Two major types of receptor for TNF with apparent molecular masses of 75 kDa and 55 kDa have been identified. To clarify whether one or both types of receptor mediate stimulation of sphingomyelinase activity, we used two different mAbs, utr-1 and htr-9. The mAb utr-1 was raised against the 75 kDa receptor and behaves as antagonist to TNFα at this receptor [19,20]. The mAb htr-9 was raised against the 55 kDa receptor and behaves as an agonist [20,21].

As shown in Table I, utr-1 had no effect on TNFα-induced changes in [^{3}H]choline lipids and water soluble metabolites. On the other hand, htr-9 mimicked the action of TNFα. htr-9 (10 μg/ml) stimulated release of [^{3}H]GroPCho/[^{3}H]PCho, decreased release of [^{3}H]choline and induced hydrolysis of sphingomyelin and phosphatidylcholine (Table I). These results suggest that TNFα stimulates the hydrolysis of sphingomyelin and phosphatidylcholine through the 55 kDa receptor.

3.3. *Effect of TNFα and mAb htr-9 on the membrane fraction of HL-60*

In order to verify whether the changes observed in intact cells are likely to be direct or indirect actions of the 55 kDa receptor, we established an assay to study sphingomyelin hydrolysis in membranes prepared from cells which had been prelabelled with [^{3}H]choline for 48 h. TNFα stimulated release of [^{3}H]GroPCho/[^{3}H]PCho in a time-dependent manner similar to that seen in intact cells but, in contrast, had no effect on [^{3}H]choline release (data not shown). The effect of TNFα and htr-9 on sphingomyelin metabolism in this cell-free system was also assessed. As shown in Fig. 2, both TNFα and htr-9 stimulated sphingomyelin hydrolysis with similar kinetics; the response was very rapid and was almost complete within 1 min of addition of TNFα or htr-9. No effect on phosphatidylcholine metabolism was observed (data not shown).

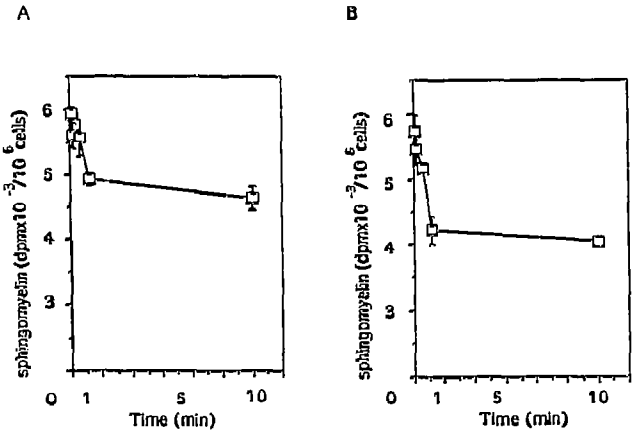

Fig. 2. Stimulation of sphingomyelin hydrolysis by TNFα and mAb htr-9. Cells were prelabelled with [^{3}H]choline and the membrane fraction prepared as described in Materials and Methods. Membranes were resuspended and stimulated with TNFα (10 nM) or mAb htr-9 (10 μg/ml) for various periods. Data are the mean ± S.E.M. of two experiments, performed in quadruplicate. A, TNFα; B, mAb htr-9.

4. DISCUSSION

In this study, the effect of mAb's raised against the 75 kDa and 55 kDa receptors for TNF were examined to clarify which receptor mediates the signalling pathway for sphingomyelinase activation. The mAb utr-1, which binds to the 75 kDa receptor and has been reported to block the ability of TNFα to interact with this receptor, had no effect on sphingomyelin hydrolysis by TNFα. In contrast, htr-9, which binds and activates the 55 kDa receptor, mimicked the ability of TNFα to stimulate formation of [^{3}H]GroPCho/[^{3}H]PCho and hydrolysis of sphingomyelin and phosphatidylcholine in intact cells and in membranes. These results suggest that the 55 kDa receptor activates sphingomyelinase to hydrolyse sphingomyelin, and phospholipases C and/or D to hydrolyse phosphatidylcholine. The observations that both TNFα and htr-9 stimulate sphingomyelinase in intact cells and in membranes with rapid kinetics suggest that this is an early and potentially important event in the signalling pathway utilized by TNFα. However, the absence of phosphatidylcholine hydrolysis in membranes suggest that this is not directly coupled to receptor activation and may be mediated subsequent to hydrolysis of sphingomyelin, for example by a ceramide-activated kinase [15,22].

In contrast to the increase in formation of [^{3}H]PCho/[^{3}H]GroPCho, TNFα and htr-9 stimulated a decrease in [^{3}H]choline in intact HL-60 cells, although this effect was not observed in membranes. The absence of a change in [^{3}H]choline levels in the membrane studies suggests that this event is secondary to hydrolysis of sphingomyelin and phosphatidylcholine, and may reflect re-incorporation of choline into lipids following their hydrolysis.

Activation of a neutral sphingomyelinase by TNFα will generate formation of ceramide. Increasing amounts of this lipid are associated with monocytic differentiation [16] and phosphorylation of the epidermal growth factor receptor [22] in HL-60 cells, possibly through a ceramide-activated protein kinase [15,22]. It is possible that generation of ceramide by the 55 kDa receptor underlies many, if not all of the actions of this TNF receptor subtype.

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